# Tumor Necrosis Factor Alpha Augments Nitric Oxide-Dependent Macrophage Cytotoxicity against *Entamoeba histolytica* by Enhanced Expression of the Nitric Oxide Synthase Gene

JIAN-YIN LIN, ROSANNE SEGUIN, KATHY KELLER, AND KRIS CHADEE*

*Institute of Parasitology of McGill University, Macdonald Campus, Ste.-Anne de Bellevue, Québec, Canada H9X 3V9*

Nitric oxide (NO measured as nitrite, $NO_2^-$) is the major effector molecule produced by activated macrophages for in vitro cytotoxicity against *Entamoeba histolytica* trophozoites. In this study, we determine whether tumor necrosis factor alpha (TNF-α) produced by activated bone marrow-derived macrophages (BMM) is involved in the induction of the inducible NO synthase gene (mac-NOS) for NO-dependent amebicidal activity. TNF-α alone did not directly induce macrophage $NO_2^-$ production to kill amebae; however, in combination with increasing concentrations of TNF-α and gamma interferon (IFN-γ), BMM amebicidal activity and $NO_2^-$ production progressively increased and showed a significant linear correlation. Antiserum to TNF-α and the NO synthase inhibitor $N^G$-monomethyl L-arginine (L-NMMA) inhibited the synergistic effects of TNF-α and IFN-γ. BMM activated with increasing concentrations of lipopolysaccharide (LPS) and IFN-γ showed a significant linear correlation between TNF-α release and $NO_2^-$ production. Antiserum to TNF-α suppressed TNF-α release, $NO_2^-$ production, and amebicidal activity by 93, 53, and 86%, respectively. L-NMMA diminished $NO_2^-$ production by 74% and macrophage amebicidal activity by 83% but had no effect on TNF-α release. Quantification by Northern (RNA) blot analyses demonstrated that IFN-γ in combination with TNF-α or LPS increased markedly the accumulation of mac-NOS and TNF-α mRNAs in a time-dependent manner with a concomitant increase in NO and TNF-α production. Peak induction of mac-NOS occurred after 24 h, whereas TNF-α mRNA was rapidly expressed after 4 h and remained stable for 48 h. Taken together, these data argue that TNF-α augments NO-dependent macrophage cytotoxicity against *E. histolytica* via elevated levels of mac-NOS mRNA expression which may be associated with the accumulation of TNF-α mRNA.

In clinical and experimental *Entamoeba histolytica* infections, macrophage-mediated effector mechanisms have been shown to be important in control of and resistance to reinfection (5, 6, 21, 25–28). Experimental intrahepatic inoculation of virulent *E. histolytica* trophozoites in congenitally athymic nude mice failed to establish an infection, but liver abscesses developed after pretreatment with silica (28). Follow-up studies using lymphocytes from patients treated for amebic liver abscess have shown that amebic proteins and concanavalin A can elicit lymphokines for the activation of naive monocytes to kill amebae (25, 26). Macrophages activated with crude lymphokines or gamma interferon (IFN-γ) in vitro killed virulent *E. histolytica* trophozoites (25, 27). We have demonstrated the acquisition of macrophage amebicidal activity with recombinant IFN-γ (rIFN-γ) alone or synergistically with lipopolysaccharide (LPS), recombinant tumor necrosis factor alpha (rTNF-α), or colony-stimulating factor 1 (6). Although amebicidal activity was associated with a strong respiratory burst, the mechanism of killing was only 45% $H_2O_2$ dependent (catalase inhibitable) and 61% inhibitable with the protease inhibitor tosyl-lysyl chlormethyl ketone. More recently (21), we have demonstrated that macrophage cytotoxicity against *E. histo-*

*lytica* is mediated primarily by NO (measured as $NO_2^-$) from L-arginine and that other reactive oxygen intermediates ($H_2O_2$ and $O_2^-$) may be cofactors for the NO effector molecule. It is evident that the mechanism of amebic killing by activated macrophages involves both oxygen-dependent and non-oxygen-dependent pathways.

TNF-α, a nonoxidative effector molecule produced by activated macrophages, has been shown to be a potent mediator of the immune response against parasitic infection (15), such as those caused by *Plasmodium yoelii* (30), *Leishmania major* (18–20, 31), *Toxoplasma gondii* (2), *Trypanosoma cruzi* (33), and *Schistosoma mansoni* (16). TNF production by macrophages is altered during *E. histolytica* infection and in response to amebic proteins in vitro (32). In combination with IFN-γ, TNF-α activates murine macrophages and human neutrophils with the capacity to kill *E. histolytica* trophozoites in vitro (6, 7). In cell-free systems, IFN-γ or TNF-α or both in high concentrations (100 to 1,000 U/ml) do not directly kill amebae or prevent parasite multiplication in culture (5). At present, little is known about the mechanism of TNF-α-induced macrophage cytotoxicity against parasites or tumor cells.

Since NO is the major effector molecule produced by activated macrophages for in vitro cytotoxicity against *E. histolytica* (21), the present study was undertaken to determine whether TNF-α is involved in the induction of the inducible NO synthase gene (mac-NOS) for NO-dependent cytotoxicity against *E. histolytica* trophozoites. The results presented here demonstrate that TNF-α augments NO production for macro-

* Corresponding author. Mailing address: Institute of Parasitology of McGill University, Macdonald Campus, 21,111 Lakeshore Road, Ste.-Anne de Bellevue, Québec, Canada H9X 3V9. Phone: (514) 398-7721. Fax: (514) 398-7857. Electronic mail address: kris@ parasit.lan.mcgill.ca.

phage cytotoxicity against *E. histolytica* via elevated levels of expression of mac-NOS mRNA which may be associated with the accumulation of TNF-α mRNA.

## MATERIALS AND METHODS

**Mice.** Female BALB/c mice, 6 to 8 weeks old and weighing approximately 18 to 20 g, were purchased from Charles River Inc. (St. Constant, Québec, Canada). They were housed in plastic cages with filter tops under pathogen-free conditions and fed water and Purina Chow ad libitum.

**Cultivation and harvesting of *E. histolytica* amebae.** Virulent axenic *E. histolytica* trophozoites (strain HM1-IMSS) originally provided by L. Diamond (National Institutes of Health, Bethesda, Md.) were passaged twice through gerbil livers and cultured in our laboratory. Culture conditions and harvesting of amebae for study were done as previously described (6).

**Reagents.** RPMI 1640 with L-glutamine was purchased from GIBCO/BRL Life Technologies (Burlington, Ontario, Canada) and was supplemented with 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 5.7 mM cysteine, and 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, Utah) (complete medium). Recombinant mouse IFN-γ and TNF-α as well as rabbit anti-mouse TNF-α polyclonal antibody were purchased from Genzyme (Cambridge, Mass.). The following were obtained from Sigma (St. Louis, Mo.): LPS (*Escherichia coli* serotype O111:B4, phenol extract), $N^G$-monomethyl L-arginine (L-NMMA), sodium nitrite, sulfanilamide, and *N*-1-naphthyl-ethylenediamine dihydrochloride.

**Preparation of bone marrow-derived macrophages (BMM).** Bone marrow cells obtained from punctured femoral bones of female BALB/c mice were incubated at 37°C in complete RPMI 1640 medium containing 15% (vol/vol) L929 cell-conditioned medium as a source of colony-stimulating factor 1. After 1 day in culture, the nonadherent cells were transferred into new culture dishes and allowed to differentiate for 7 days. Macrophages were made quiescent by being cultured in colony-stimulating factor 1-free medium for 18 h prior to being used (8).

**Assessment of macrophage amebicidal activity.** Macrophages were pretreated as indicated. Macrophages ($2 \times 10^6$ cells per ml) and *E. histolytica* trophozoites ($2 \times 10^4$ cells per ml) suspended in complete RPMI 1640 medium in plastic tubes were centrifuged at $150 \times g$ for 10 min and incubated at 37°C in 5% $CO_2$ for 6 h. After incubation, the tubes were placed on ice and viability of the trophozoites was assayed by trypan blue exclusion. The data are expressed as percent amebic viability.

**Measurement of $NO_2^-$ production.** Culture supernatants (50 μl) were assayed for $NO_2^-$ by the Griess reaction according to the microassay recently described by Ding et al. (10). Briefly, an equal volume of Griess reagent (1% sulfanilamide–0.1% *N*-1-naphthyl-ethylenediamine dihydrochloride in 2.5% $H_3PO_4$) was incubated with macrophage supernatants for 10 min at room temperature, and the $A_{550}$ was measured with an enzyme-linked immunosorbent assay (ELISA) reader (Biotecx Instruments, Mandel Scientific, Ontario, Canada). $NO_2^-$ concentration was determined by using sodium nitrite as a standard. Data are expressed as total micromolar $NO_2^-$ produced by $10^6$ cells at the times indicated in the table and figures.

**TNF-α ELISA.** TNF-α production was measured by an ELISA method described elsewhere (29). Briefly, 50 μl of hamster anti-mouse TNF-α monoclonal antibody (Genzyme), diluted 1:2,000 in pH 9.6 carbonate buffer, was incubated in each well of MicroTest ELISA plates (Falcon) at 4°C overnight. The plates were washed with phosphate-buffered saline (PBS)-Tween (0.01 M PBS–0.05% Tween 20; washing buffer) and shaken to remove residual liquid. Into each well, 100 μl of 2% bovine serum albumin (BSA) (BSA in 0.01 M PBS; blocking buffer) was added and the plates were incubated at 37°C for 1 h. Plates were washed, and 50 μl of standard recombinant murine TNF-α (Genzyme) in blocking buffer or 50 μl of macrophage culture supernatants was put into each well and incubated at 37°C for 1 h. After washing, 50 μl of rabbit anti-mouse TNF-α polyclonal antibody (Genzyme) diluted 1:200 in blocking buffer was added and the plates were incubated at 37°C for 1 h, washed, and incubated with 50 μl of goat anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) at a 1:3,000 dilution for 1 h. The plates were washed three times with washing buffer and once with PBS. Finally, 100 μl of ABTS (Bio-Rad) was added to each well. The optical density was measured 10 to 15 min later with an ELISA reader. The TNF-α concentration was measured by using recombinant mouse TNF-α as a standard (128 to 2 U/ml). Data are expressed as units per milliliter per $10^6$ cells.

**Preparation of RNA and Northern (RNA) blot analysis.** Total RNA was isolated from BMM ($10^7$ cells per 10 ml) with RNAzol B (TelTest Inc., Friendswood, Tex.), which is a modification of the guanidium-phenol-chloroform method (3). Samples of total RNA (10 μg) were denatured for 1 h at 50°C in the presence of glyxoxal, and 1 μg of ethidium bromide was added to each sample before electrophoresis in a 1% agarose gel. After electrophoresis, RNA was transferred to a Hybond-N nylon membrane (Amersham Canada Ltd., Oakville, Ontario, Canada). The membrane was UV cross-linked and prehybridized for 3 h at 42°C with a solution containing $5 \times$ SSPE ($1 \times$ SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), $5 \times$ Denhardt solution, 50% formamide, 0.1% sodium dodecyl sulfate, and 250 μg of denatured salmon sperm DNA per ml. Hybridization was performed at 42°C overnight (18 h) with probes purified from agarose and nick translated in the presence of 125 μCi of [$^{32}$P]dCTP (ICN Biochemicals, Québec, Canada). The blot was then washed once at room temperature and twice at 55°C in $0.5 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 30 min each. Autoradiography was performed at $-70°C$ in cassettes with an intensifying screen, using Kodak XAR-5 films (Eastman Kodak Company, Rochester, N.Y.). The inducible mac-NOS mouse probe was the 4.1-kb *Not*I fragment from pmmac-NOS kindly supplied by Charles J. Lowenstein, Johns Hopkins University School of Medicine, Baltimore, Md. The TNF-α probe was the 1.5-kb *Pst*I fragment from pmTNF-1 from W. Fiers, University of Gent, Gent, Belgium. To ensure that equal amounts of RNA were analyzed, blots were stripped, rehybridized with a radiolabeled cDNA probe for actin (1.25-kb *Pst*I fragment of pBA-1), washed, and again subjected to autoradiography. For quantification by scanning densitometry, multiple exposures were used to ensure that all signals were within the linear response range of the film.

**Statistical analysis.** All experiments were performed two or three times, and the results were analyzed by Student's *t* test and regression analysis. A value of $P < 0.05$ is regarded as statistically significant.

## RESULTS

**rTNF-α synergizes with rIFN-γ in macrophage-mediated killing of *E. histolytica* trophozoites via the induction of NO.** We have previously shown that TNF-α in combination with
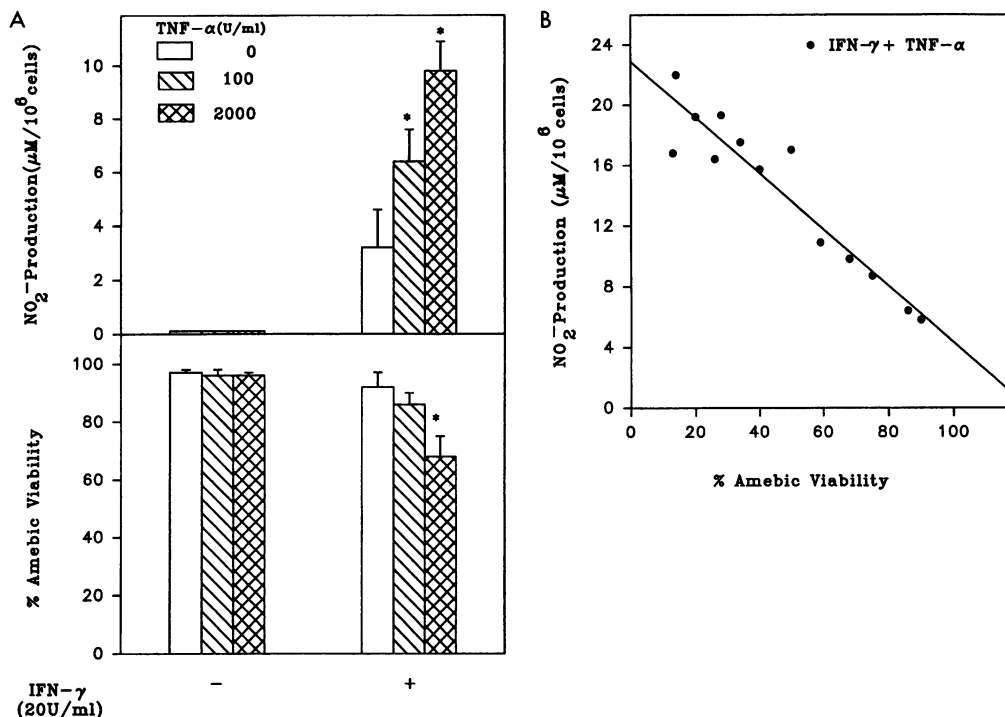
FIG. 1. (A) Synergistic effects of TNF-α and IFN-γ on both macrophage amebicidal activity and $NO_2^-$ release in vitro. BMM were pretreated for 48 h with TNF-α at the indicated concentrations alone or in the presence of IFN-γ before amebicidal activity and $NO_2^-$ production were measured. Data are the means ± standard deviations (SD) of triplicate experiments ($n = 12$). * , $P < 0.01$ compared with untreated TNF-α control. (B) Relationship between macrophage amebicidal activity and $NO_2^-$ production. BMM were pretreated for 48 h with graded doses of IFN-γ (20 or 100 U/ml) plus TNF-α (100, 1,000, or 2,000 U/ml) ($r = 0.7443; P < 0.01; n = 13$) before amebicidal activity and $NO_2^-$ production were measured in parallel.

IFN-γ synergistically induced macrophages and neutrophils with potent amebicidal activity (6, 7), and more recently, we have further demonstrated that NO is the major effector molecule released by activated macrophages for the killing of amebae (21). Experiments were therefore carried out to determine whether the synergistic effect of TNF-α with IFN-γ on the induction of macrophage amebicidal activity is involved in the production of NO in vitro. As shown in Fig. 1A, TNF-α alone even at a high concentration (2,000 U/ml) did not induce BMM amebicidal activity and $NO_2^-$ release. Similarly, macrophages pretreated with a low concentration of IFN-γ (20 U/ml) alone also failed to kill the parasites and to produce appreciable levels of $NO_2^-$ (<3.2 μM/$10^6$ cells). In contrast, IFN-γ-treated macrophages in the presence of TNF-α were highly effective in killing the parasites, concomitant with a corresponding increase in $NO_2^-$ production. The synergistic effect of TNF-α with IFN-γ was highly dependent on the concentration of TNF-α. For example, with 20 U of IFN-γ per ml, 100 U of TNF-α per ml had no significant effect on the induction of macrophage amebicidal activity ($P > 0.05$) but significantly enhanced $NO_2^-$ production ($P < 0.01$); however, with 2,000 U of TNF-α per ml both amebicidal activity and $NO_2^-$ release by IFN-γ-primed macrophages were significantly increased (Fig. 1A, $P < 0.01$).

In order to establish more precisely the synergy of IFN-γ and TNF-α for the induction of macrophage amebicidal activity and $NO_2^-$ production, we examined this relationship by linear regression analysis. As shown in Fig. 1B, there was a significant correlation ($r = 0.7443; P < 0.01; n = 13$) between $NO_2^-$ production and amebicidal activity with increasing concentrations of IFN-γ and TNF-α. When the data were

analyzed by using 20 U of IFN-γ per ml with increasing concentrations of TNF-α (100, 1,000, and 2,000 U/ml), similar results were obtained ($r = 0.9226; P < 0.01; n = 15$; results not shown). These data indicate that the synergistic effect of IFN-γ and TNF-α in the induction of macrophage amebicidal activity involves the production of NO in vitro.

To determine the specificity of TNF-α in the induction of amebicidal activity and $NO_2^-$ production by macrophages activated by IFN-γ plus TNF-α, we investigated the neutralizing effect of antiserum to TNF-α in this reaction. As shown in Fig. 2, activated BMM in the presence of rabbit anti-TNF-α serum resulted in 86 and 61% inhibition of amebicidal activity and $NO_2^-$ production, respectively, compared with those of activated BMM or preimmune rabbit serum (1% [vol/vol]). Similarly, in the presence of 500 μM L-NMMA, a competitive inhibitor of L-arginine metabolism by the enzyme of the NO pathway, maximal inhibitory effects on parasite killing (93% live amebae) and $NO_2^-$ production (<2.2 μM/$10^6$ cells) were noted. These results clearly demonstrate that the synergistic effect of IFN-γ plus TNF-α in the induction of macrophage amebicidal activity via $NO_2^-$ production is specific for TNF-α.

**An autocrine role for TNF-α in the induction of macrophage amebicidal activity and NO production by IFN-γ plus LPS.** To further compare the relative roles of TNF-α with NO for the induction of macrophage amebicidal activity, experiments were designed to determine the relationship between TNF-α release and $NO_2^-$ production by IFN-γ-plus-LPS-activated macrophages. BMM treated with LPS alone resulted in a dose-dependent stimulation of TNF-α release ($P < 0.01$) that plateaued at 100 ng/ml but produced relatively low levels of $NO_2^-$ (<2 μM; Fig. 3A). Macrophages pretreated with 20 U
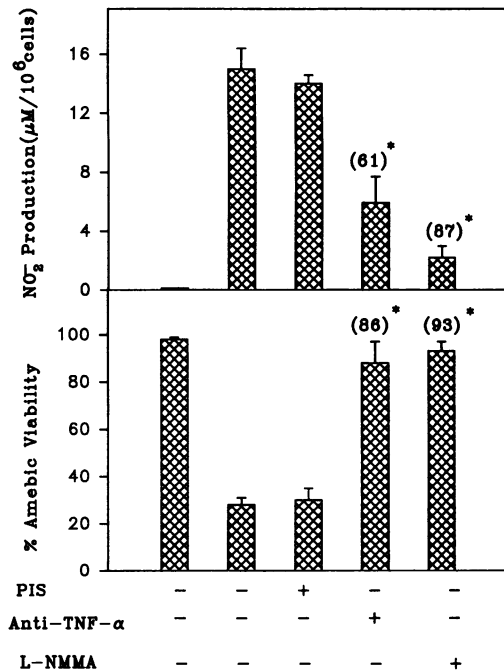
FIG. 2. Neutralizing antiserum to TNF-α reverses amebicidal activity and $NO_2^-$ production by macrophages activated by IFN-γ plus TNF-α. BMM were pretreated for 48 h with IFN-γ (100 U/ml) plus TNF-α (1,000 U/ml) alone or in the presence of 1% (vol/vol) preimmune rabbit serum (PIS) and rabbit anti-TNF-α serum 1% (vol/vol) or L-NMMA (500 μM) before amebicidal activity and $NO_2^-$ production were measured. Data are the means ± SD of duplicate experiments (n = 6). Values in parentheses show percent inhibition of the response relative to that of the IFN-γ plus TNF-α control. *, P < 0.01 compared with the IFN-γ plus TNF-α control.

of IFN-γ per ml alone failed to stimulate TNF-α release and $NO_2^-$ production. However, in the presence of increasing concentrations of LPS (10, 100, or 1,000 ng), TNF-α release (P < 0.01) was enhanced, concomitant with increased $NO_2^-$ production (>6 μM; P < 0.01) (Fig. 3A). There was a positive correlation between TNF-α release and $NO_2^-$ production over 24 h (Fig. 3B; r = 0.871; P < 0.01; n = 9). These data demonstrate that TNF-α performs an essential autocrine function in the production of NO by IFN-γ-plus-LPS-activated macrophages.

To test the relationship between TNF-α, $NO_2^-$ production, and amebicidal activity, BMM were pretreated for 24 h with IFN-γ (100 U/ml) plus LPS (100 ng/ml) in the presence of rabbit anti-mouse TNF-α (1% [vol/vol]), L-NMMA (500 μM), or both before the TNF-α, $NO_2^-$, and amebicidal activities were measured. As shown in Table 1, neutralizing anti-TNF-α antibody inhibited TNF-α release, $NO_2^-$ production, and amebicidal activity by 93, 53, and 86%, respectively (P < 0.01). L-NMMA also diminished $NO_2^-$ production by 74% and amebicidal activity by 83% (P < 0.01) but had no effect on TNF-α release. Antiserum to TNF-α and L-NMMA together abrogated TNF-α release, $NO_2^-$ production, and amebicidal activity by 93, 78, and 100%, respectively. These results unequivocally demonstrate an autocrine role for TNF-α in stimulating the production of NO concomitant with increased amebicidal activity by macrophages activated by IFN-γ plus LPS and the importance of TNF-α in mediating NO-dependent amebicidal activity.

**Cooperative induction of both mac-NOS and TNF-α mRNAs by IFN-γ plus TNF-α and IFN-γ plus LPS.** The foregoing experiments demonstrated that TNF-α augments NO production for macrophage cytotoxicity against *E. histolytica* trophozoites. Thus, in order to explore the mechanisms involved, experiments were carried out to investigate the induction of
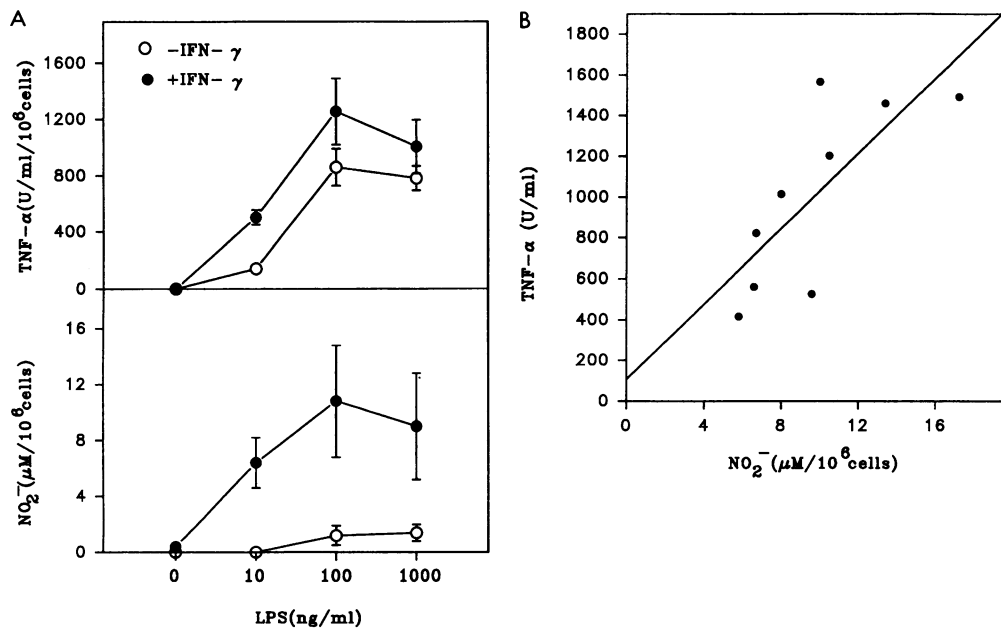


FIG. 3. (A) Induction of TNF-α release and $NO_2^-$ production by macrophages pretreated with IFN-γ and LPS. BMM were pretreated for 24 h with increasing concentrations of LPS alone or in the presence of IFN-γ (20 U/ml) before TNF-α release was measured by ELISA and $NO_2^-$ production was measured as described in Materials and Methods. Data are the means ± SD of duplicate experiments (n = 6). (B) Correlation between TNF-α release and $NO_2^-$ production by IFN-γ-plus-LPS-activated macrophages. BMM were pretreated for 24 h with various doses of LPS (10, 100, or 1,000 ng/ml) in the presence of IFN-γ (20 U/ml) before TNF-α release and $NO_2^-$ production were measured in parallel (r = 0.871; P < 0.01; n = 9).

TABLE 1. Effects of rabbit anti-mouse TNF-α and L-NMMA on TNF-α release, $NO_2^-$ production, and amebicidal activity by activated BMM[a]

| Treatment | TNF-α (U/ml/$10^6$ cells) | $NO_2^-$ (μM/$10^6$ cells) | % Amebic viability |
|---|---|---|---|
| None | 0 | 0.2 ± 0.3 | 98 ± 1 |
| IFN-γ + LPS | 1,262 ± 122 | 15.8 ± 2.3 | 34 ± 8 |
| IFN-γ + LPS + anti-TNF-α | 83 ± 18 (93) | 7.4 ± 1.6 (53) | 89 ± 5 (86) |
| IFN-γ + LPS + L-NMMA | 1,301 ± 78 (0) | 4.1 ± 0.3 (74) | 87 ± 4 (83) |
| IFN-γ + LPS + anti-TNF-α + L-NMMA | 91 ± 12 (93) | 3.4 ± 0.3 (78) | 98 ± 1 (100) |

[a] BMM were pretreated for 24 h with IFN-γ (100 U/ml) plus LPS (100 ng/ml) alone or in the presence of rabbit anti-mouse TNF-α (1%, vol/vol) and/or L-NMMA (500 μM) before TNF-α, $NO_2^-$ production, and amebicidal activity were measured. Data are the means ± SD of duplicate experiments (n = 6). Values in parentheses are percents inhibition of the response relative to that of the IFN-γ plus LPS control. Preimmune rabbit serum had no inhibitory effect.

both mac-NOS and TNF-α mRNAs by IFN-γ plus TNF-α/LPS and the relationship between them. As shown in Fig. 4, BMM stimulated for 48 h with IFN-γ or TNF-α alone induced detectable levels of mac-NOS and TNF-α mRNA expression, whereas cells treated with LPS alone did not express TNF-α mRNA levels, although detectable levels of mac-NOS mRNA were induced. However, when combined with IFN-γ, TNF-α
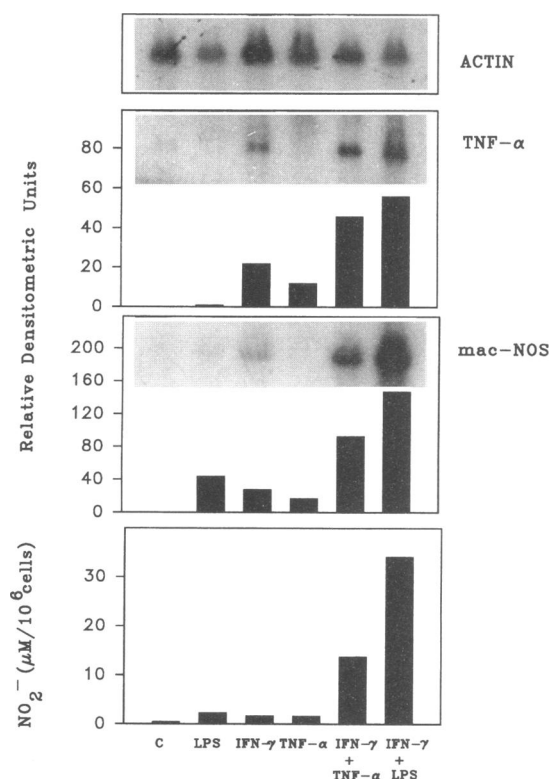


FIG. 4. Induction of mac-NOS and TNF-α mRNAs in macrophages by various stimuli. BMM were stimulated with medium (c), LPS (100 ng/ml), IFN-γ (100 U/ml), TNF-α (100 U/ml), IFN-γ plus TNF-α, or IFN-γ plus LPS for 48 h. $NO_2^-$ production in culture supernatants was then measured and total RNA was isolated. The levels of mac-NOS, TNF-α, and actin mRNAs were assessed by Northern analysis as described in Materials and Methods. Results of scanning densitometric analysis of the Northern blot are presented as a histogram. The relative levels of mac-NOS and TNF-α mRNAs were determined after normalization to the respective actin signal to account for variability in the amounts of RNA. Lane designations are identical for the blot and the histogram. Similar results were obtained in three separate experiments.

or LPS increased markedly the accumulation of mac-NOS and TNF-α mRNAs; in particular, mac-NOS mRNA levels were increased many times more than would be caused by the additive levels of each cytokine alone. To determine whether the enhanced mac-NOS mRNA levels resulted in a corresponding increase in NO production, we determined the cumulative nitrite levels as a measure of NO release into cell culture supernatants. As shown in Fig. 4, NO production levels paralleled mRNA levels at 48 h after stimulation. In contrast, TNF-α production was maximal after 24 h (Fig. 3A) and remained unchanged after 48 h. These data clearly indicate that either exogenous or endogenous TNF-α in combination with IFN-γ acts synergistically for the induction of mac-NOS and TNF-α mRNA for NO production.

To assess the kinetics by which mac-NOS and TNF-α mRNAs were induced, BMM were stimulated with IFN-γ plus TNF-α/LPS for various lengths of time. As shown in Fig. 5, accumulation of mac-NOS mRNA occurred after 4 h of stimulation with IFN-γ plus TNF-α/LPS, peaked at 24 h, and decreased slightly by 48 h. High levels of TNF-α mRNA were observed at 4 h after stimulation with IFN-γ plus LPS, after which levels declined at a considerably lower rate and no longer decreased at a later time point. However, accumulation of TNF-α mRNA by IFN-γ plus TNF-α stimulation was consistent during 4 to 48 h of stimulation. LPS, IFN-γ, or TNF-α alone caused a modest accumulation of mac-NOS mRNA after 24 h, but the level decreased thereafter. TNF-α mRNA was maximally expressed after 4 h in response to LPS stimulation but returned to basal levels after 48 h, whereas TNF-α mRNA levels remained consistently low in response to IFN-γ or TNF-α stimulation (Fig. 5). These data indicate that the cooperative effects of IFN-γ plus TNF-α/LPS on induction of mac-NOS and TNF-α mRNAs occurred in a time-dependent manner. Taken together, these results suggest that TNF-α augments NO-dependent macrophage cytotoxicity against E. histolytica trophozoites through elevated levels of mac-NOS mRNA expression, which may be associated with the accumulation of TNF-α mRNA.

## DISCUSSION

We have previously documented that TNF-α in combination with IFN-γ can endow murine macrophages and human neutrophils with the capacity to kill E. histolytica trophozoites in vitro (6, 7). More recently, we extended these observations to demonstrate that macrophage cytotoxicity against E. histolytica trophozoites is mediated by NO from L-arginine (21). In this study, we determined whether TNF-α produced by activated macrophages was involved in the induction of mac-NOS mRNA for NO-dependent cytotoxicity against E. histolytica. Experiments reported here argue that TNF-α augments NO-

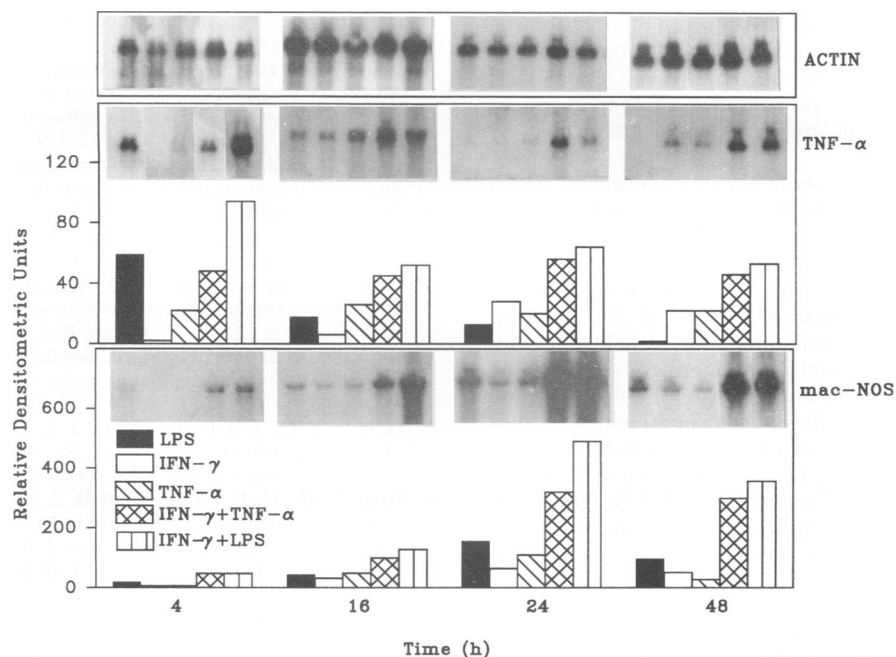FIG. 5. Kinetics of induction of both mac-NOS and TNF-α mRNAs by IFN-γ plus TNF-α/LPS stimulation. BMM were stimulated with IFN-γ (100 U/ml) plus TNF-α (100 U/ml)/LPS (100 ng/ml) for 4, 16, 24, and 48 h. Levels of mac-NOS and TNF-α mRNAs were then determined by Northern blot analysis. Results of scanning densitometric analysis of the Northern blot are presented as a histogram. The relative levels of mac-NOS and TNF-α mRNAs were determined as described in the legend to Fig. 4. Lane designations are identical for the blot and the histogram. Similar results were obtained in two separate experiments.

dependent macrophage cytotoxicity against *E. histolytica* via elevated levels of expression of mac-NOS mRNA, which may be associated with accumulation of TNF-α mRNA by the following observations: (i) IFN-γ synergizes with TNF-α in macrophage-mediated killing of *E. histolytica* via the induction of NO from L-arginine, (ii) TNF-α plays an autocrine role for NO-dependent amebicidal activity by IFN-γ-plus-LPS-activated macrophages, and (iii) IFN-γ in combination with exogenous or endogenous TNF-α acts synergistically to increase markedly the accumulation of both mac-NOS and TNF-α mRNAs, with a concomitant increase in NO production.

It has been shown previously that both synthesis of NO and induction of macrophage cytotoxicity require multiple signals delivered to macrophages in a defined sequence of which IFN-γ is the major priming signal (11, 24). The sequential exposure to IFN-γ and LPS is probably the best-studied model. Other cytokines that have been reported to play a potential role in macrophage activation include granulocyte macrophage-colony-stimulating factor and TNF-α (12, 15, 17, 23). In this study, we have shown that exogenously added TNF-α plays an essential role in the induction of macrophage amebicidal activity by IFN-γ via regulation of the L-arginine-dependent production of NO by these cells. This conclusion is based on the following observations. TNF-α alone did not directly induce synthesis of NO to kill amebae. However, in combination with IFN-γ, mac-NOS mRNA was expressed fourfold after 24 h, concomitant with increased $NO_2^-$ production and enhanced macrophage amebicidal activity. L-NMMA, an NO synthase inhibitor, inhibited both macrophage amebicidal activity and $NO_2^-$ production. Finally, neutralizing antiserum to TNF-α reversed the synergistic effect of TNF-α and IFN-γ on the induction of macrophage-mediated killing of amebae and NO synthesis. These data indicate that TNF-α is a second signal for triggering of NO-dependent macrophage cytotoxicity

against *E. histolytica*. Although these experiments demonstrate a clear role for TNF-α in triggering IFN-γ-primed macrophages for killing amebae, there is a difference between our findings and previous reports of the synergistic effects of TNF-α and IFN-γ. That is, TNF-α by itself is not cytotoxic to amebae (5), whereas it is directly toxic to some tumor cells (13) and *T. cruzi* (9). In this study, we found that there is a close correlation between $NO_2^-$ production and the level of TNF-α release from IFN-γ-plus-LPS-activated macrophages. This relationship suggests that LPS may stimulate TNF-α release from IFN-γ-primed macrophages and that the released TNF-α acts as a second signal in an autocrine manner to trigger the production of NO by these cells. To test this hypothesis, we attempted to neutralize the triggering capacity of TNF-α by using a polyclonal antiserum to TNF-α. This antiserum almost completely inhibited the release of endogenous TNF-α from (93% inhibition) and amebicidal activity of these cells. It is apparent that when LPS stimulates TNF-α extracellular release, antiserum to TNF-α is able to block it. In light of this concept, the autocrine hypothesis is correct. This finding is consistent with previous data showing an important autocrine role for TNF-α in enhancing the production of NO by primed or activated macrophages (12). We also found that $NO_2^-$ production by IFN-γ-plus-LPS-activated macrophages could not be completely suppressed by antiserum to TNF-α (53% inhibition). It is possible that both TNF-α and LPS act as triggers for IFN-γ-primed macrophages independently through a receptor(s) that stimulates a common signalling pathway. These findings indicate two alternative models for how TNF-α and LPS may be associated with triggering macrophage activation to produce NO, the major effector molecule for killing amebae. On the other hand, the evidence that L-NMMA abolished $NO_2^-$ production (73% inhibition) and amebicidal activity (83% inhibition) without impairing the

release of TNF-α further supports the concept of a central role of NO-dependent or TNF-α-independent macrophage cytotoxicity against *E. histolytica* and suggests that the induction of NO synthase is a necessary precondition for expression of NO-dependent or TNF-α-independent amebicidal activity by activated macrophages.

At present, little is known about the mechanisms by which TNF-α induces NO synthesis by macrophages. NO synthesis in macrophages is mediated by a $Ca^{2+}$-independent NO synthase, which is induced after exposure to cytokine and/or LPS (34), and extracellular L-arginine is a requirement for the production of NO from activated macrophages (1, 14, 21). A recent study (1) has demonstrated that stimulation of J774 cells with LPS alone resulted in a substantial enhancement of L-arginine transport, followed by the accumulation of $NO_2^-$ in the culture medium. However, following activation with LPS plus IFN-γ, even though $NO_2^-$ production was increased, L-arginine uptake was not augmented (1). Lorsbach et al. (22) have shown that the expression of mac-NOS in RAW 264.7 cells costimulated with IFN-γ and LPS is greatly increased compared with that induced by LPS alone. There is only one report of the cooperative induction of mac-NOS mRNA by IFN-γ and TNF-α (4). In our study, we have shown that in combination with IFN-γ, TNF-α and LPS increased markedly the accumulation of mac-NOS and TNF-α mRNAs in close parallel with the production of NO. Interestingly, TNF-α mRNA levels remained consistently high throughout the 4- to 48-h stimulation period whereas mac-NOS was maximally expressed after 24 h. These results suggest that macrophages require sustained expression of TNF-α mRNA in order to maintain a level of TNF-α in culture supernatants sufficient to trigger a high level of mac-NOS mRNA expression. In addition, because TNF-α uses the p55 receptor to stimulate mac-NOS mRNA (4), the cooperative effect of IFN-γ and TNF-α might be involved in selective modulation of TNF receptor gene expression.

In conclusion, results presented here argue that TNF-α augments NO-dependent macrophage cytotoxicity against *E. histolytica* trophozoites through elevated levels of expression of mac-NOS mRNA, which may be associated with the accumulation of TNF-α mRNA. This implies that TNF-α may also be an important regulator of macrophage activation in vivo. This hypothesis is supported by our findings that basal and stimulated TNF-α production by abscess macrophages and macrophages distant from the abscess in a gerbil model of amebic liver abscess is modulated during the course of an infection (32), which may play an important role in the regulation of macrophage amebicidal activity. Regulation of macrophage activation by TNF-α has important implications for immunotherapy protocols involving exogenously administered IFN-γ.

## ACKNOWLEDGMENTS

## REFERENCES

1. **Bogle, R. G., A. R. Baydoun, J. D. Pearson, S. Moncada, and G. E. Mann.** 1992. L-Arginine transport is increased in macrophages generating nitric oxide. Biochem. J. **284:**15–18.
2. **Chang, H. R., G. E. Grau, and J. C. Pechere.** 1990. Role of TNF-α and IL-1 in infections with *Toxoplasma gondii*. Immunology **69:**33–37.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single step method of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Deng, W., B. Thiel, C. S. Tannenbaum, T. A. Hamilton, and D. J. Stuehr.** 1993. Synergistic cooperation between T cell lymphokines for induction of the nitric oxide synthase gene in murine peritoneal macrophages. J. Immunol. **151:**322–329.
5. **Denis, M., and K. Chadee.** 1988. Immunopathology of *Entamoeba histolytica* infections. Parasitol. Today **4:**247–252.
6. **Denis, M., and K. Chadee.** 1989. Cytokine activation of murine macrophages for in vitro killing of *Entamoeba histolytica* trophozoites. Infect. Immun. **57:**1750–1756.
7. **Denis, M., and K. Chadee.** 1989. Human neutrophils activated by interferon-γ and tumor necrosis factor-α kill *Entamoeba histolytica* trophozoites in vitro. J. Leukocyte Biol. **46:**270–274.
8. **Descoteaux, A., and G. Matlashewski.** 1989. c-*fos* and tumor necrosis factor gene expression in *Leishmania donovani*-infected macrophages. Mol. Cell. Biol. **9:**5223–5227.
9. **DeTitto, E. H., J. R. Catterall, and J. S. Remington.** 1986. Activity of recombinant tumor necrosis factor on *Toxoplasma gondii* and *Trypanosoma cruzi*. J. Immunol. **137:**1342–1345.
10. **Ding, A. H., C. F. Nathan, and D. J. Stuehr.** 1988. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. J. Immunol. **141:**2407–2412.
11. **Drapier, J. C., J. Wietzerbine, and J. B. Hibbs.** 1988. Interferon-γ and tumor necrosis factor induce the L-arginine-dependent cytotoxic effector mechanism in murine macrophages. Eur. J. Immunol. **18:**1587–1595.
12. **Green, S. J., R. M. Crawford, J. T. Hockmeyer, M. S. Meltzer, and C. A. Nacy.** 1990. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. J. Immunol. **145:**4290–4297.
13. **Heidenreich, S., M. Weyers, J. H. Gong, H. Sprenger, M. Nain, and D. Gemsa.** 1988. Potentiation of lymphokine-macrophage activation by tumor necrosis factor-α. J. Immunol. **140:**1511–1518.
14. **Hibbs, J. B., Z. Vavrin, and R. R. Taintor.** 1987. L-Arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J. Immunol. **138:**550–565.
15. **James, S. L.** 1991. The effector function of nitrogen oxides in host defense against parasites. Exp. Parasitol. **73:**223–226.
16. **James, S. L., J. Glaven, S. Goldenberg, M. S. Meltzer, and E. Pearce.** 1990. Tumor necrosis factor (TNF) as a mediator of macrophage helminthotoxic activity. Parasite Immunol. **12:**1–13.
17. **Liew, F. Y., and F. E. G. Cox.** 1991. Nonspecific defence mechanism: the role of nitric oxide. Parasitol. Today **7:**A17–A21.
18. **Liew, F. Y., Y. Li, and S. Millott.** 1990. Tumor necrosis factor (TNF-α) in leishmaniasis. II. TNF-α-induced macrophage leishmanicidal activity is mediated by nitric oxide from L-arginine. Immunology **71:**556–559.
19. **Liew, F. Y., Y. Li, and S. Millott.** 1990. Tumor necrosis factor-α synergizes with IFN-γ in mediating killing of *Leishmania major* through the induction of nitric oxide. J. Immunol. **145:**4306–4310.
20. **Liew, F. Y., C. Parkinson, S. Millott, A. Severn, and M. Carrier.** 1990. Tumor necrosis factor (TNF-α) in leishmaniasis. I. TNF-α mediated host protection against cutaneous leishmaniasis. Immunology **69:**570–573.
21. **Lin, J. Y., and K. Chadee.** 1992. Macrophage cytotoxicity against *Entamoeba histolytica* trophozoites is mediated by nitric oxide from L-arginine. J. Immunol. **148:**3999–4005.
22. **Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell.** 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor killing. J. Biol. Chem. **269:**1908–1913.
23. **Nacy, C. A., B. J. Nelson, M. S. Meltzer, and S. J. Green.** 1991. Cytokines that regulate macrophage production of nitrogen oxides and expression of antileishmanial activities. Res. Immunol. **142:**573–576.
24. **Ruco, L. P., and M. S. Meltzer.** 1978. Macrophage activation for tumor cytotoxicity: development of macrophage cytotoxic activity requires completion of short-lived intermediary reactions. J. Immunol. **121:**2034–2040.
25. **Salata, R. A., A. Martinez-Palomo, H. W. Murray, L. Conales, N.**

Trevino, E. S. Segovia, C. F. Murphy, and J. I. Ravdin. 1986. Patients treated for amebic liver abscess develop cell-mediated immune responses effective in vitro against *Entamoeba histolytica*. J. Immunol. **136:**2633–2641.

26. Salata, R. A., H. W. Murray, B. Y. Rubin, and J. I. Ravdin. 1987. The role of gamma interferon in the generation of human macrophages cytotoxic for *Entamoeba histolytica* trophozoites. Am. J. Trop. Med. Hyg. **37:**72–78.

27. Salata, R. A., R. D. Pearson, and J. I. Ravdin. 1985. Interaction of human leukocytes and *Entamoeba histolytica*: killing of virulent amebae by the activated macrophages. J. Clin. Invest. **76:**491–499.

28. Stern, J. J., R. Graybill, and D. J. Drutz. 1984. Murine amebiasis: the role of the macrophages in host defense. Am. J. Trop. Med. Hyg. **33:**372–380.

29. Tachibana, K., G. J. Chen, D. S. Huang, P. Scuderi, and R. R. Watson. 1992. Production of tumor necrosis factor-α by resident and activated murine macrophages. J. Leukocyte Biol. **51:**251–255.

30. Taverne, J., J. Ravernier, W. Fiers, and J. H. L. Playfair. 1987. Recombinant tumor necrosis factor inhibits malaria parasites in vivo but not in vitro. Clin. Exp. Immunol. **67:**1–4.

31. Titus, R. G., B. Sherry, and A. Cerami. 1989. Tumor necrosis factor plays a protective role in experimental cutaneous leishmaniasis. J. Exp. Med. **170:**2097–2104.

32. Wang, W., K. Keller, and K. Chadee. 1992. Modulation of tumor necrosis factor production by macrophages in *Entamoeba histolytica* infection. Infect. Immun. **60:**3169–3174.

33. Wirth, J. J., and F. Kierszenbaum. 1988. Recombinant tumor necrosis factor enhances macrophage destruction of *Trypanosoma cruzi* in the presence of bacterial endotoxin. J. Immunol. **141:**286–288.

34. Xie, Q. W., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso, and C. Nathan. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science **256:**225–228.